Identification and Investigation of the Centrosome Localization Domain of Mps1

A Senior Honors Thesis

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by

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Abstract

The centrosome is a single copy organelle that is responsible for organizing the microtubule network throughout the cell cycle. The centrosome also regulates the cell cycle, organizes the mitotic spindle, and is required for cytokinesis. In fact, the organelle is a major factor in the conservation of genomic integrity. Mps1 is a family of protein kinases originally identified in *Saccharomyces cerevisiae*. Much is known about the functions of the human orthologue hMps1; it is involved in the mitotic spindle checkpoint, centrosome duplication, and cytokinesis. It is localized to the centrosome throughout the cell cycle, but the method by which it is targeted to centrosomes is unknown.

In this study we identify a conserved domain of hMps1 and propose that is involved in targeting the protein to the centrosome. We show that it is sufficient for centrosome localization and are working on determining its necessity. Within the identified domain is a tyrosine residue (Y91) that is completely conserved in vertebrate species. Tyrosine residues are often important because they can be phosphorylated and are sometimes involved in the regulation of a protein's function. Here we demonstrate that phosphorylation of Y91 is indeed involved in the regulation of centrosome localization and propose methods to determine how it might regulate that event. We also utilize the two-hybrid system to determine proteins that bind to the centrosome localization domain (CLD). We have identified five proteins from the screen and further investigated one of them, VDAC3. Using GFP-fusion proteins we provide evidence that VDAC3 can indeed localize to centrosomes and are testing its interaction with hMps1.

This work is significant because centrosome misregulation can lead to serious cellular defects. In fact, abnormal centrosome numbers have been found in a number of tumors, and are especially prevalent in breast cancer. Therefore, centrosomal defects could potentially be a catalyst for the progression of cancer; and because of its function in centrosome duplication and cytokinesis, defects in Mps1 itself could be a factor in the development of human tumors. It is therefore essential to understand the mechanism of Mps1 centrosome localization to explain problems that occur in cancers containing chromosome defects.

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Chapter 1

Introduction to the Centrosome and Mps1

The centrosome is the microtubule-organizing center (MTOC) found in animal cells. Each centrosome consists of two centrioles surrounded by a pericentriolar matrix (PCM) which serves to nucleate microtubules (1). The centrioles are cylindrical structures composed of nine sets of triplet microtubules, and are structurally distinct from one another. The older centriole in the pair, called the mother centriole, is decorated with distal and sub-distal appendages. The younger centriole, called the daughter centriole, does not possess these structures. Originally discovered by Boveri in 1888, the centrosome is primarily responsible for segregating the sister chromatids during cell division. It is also an important cell cycle regulator required for the decision to enter the cell cycle and undergo transition from G1 to S phase (2, 3), and for the assembly and function of the mitotic spindle (4). It is a single copy organelle that must be duplicated every round of cell division and its duplication occurs during the S phase in mammalian cells (5, 6). It is imperative that the centrosome is duplicated and only duplicated once per cell cycle in order to form a strictly bipolar mitotic spindle during cell division (7, 8) and regulate cytokinesis (9, 10) in order to form two identical daughter cells. When centrosomes are over-duplicated, duplicated more than once per cell cycle, they cannot properly segregate chromosomes. When this happens the resulting cells will not contain the correct number of chromosomes, a condition known as aneuploidy, and will not be able to function correctly. When the centrosome is unable to ensure proper chromosome segregation and cell division during mitosis, severe problems, such as aneuploidy, can

occur. The seriousness of defects like aneuploidy are made evident by the fact that they are observed in human tumors (8).

Mps1 is a dual specificity protein kinase that has been shown to be involved in centrosome duplication, the mitotic spindle checkpoint, and cytokinesis. Mps1 was originally discovered in budding yeast, *Saccharomyces cerevisiae*, where it was found to be an essential gene involved in spindle pole body (SPB) duplication (11) and was later learned to function in the spindle checkpoint (12, 13). The SPB is the MTOC in yeast; it has functional similarity to the mammalian centrosome although its structure is quite different. Since Mps1 was discovered, researchers have found orthologues in higher eukaryotes including humans (14). Mps1 is an interesting candidate for research because it is involved in many aspects of the centrosome's function, and specifically those functions that, when there are errors, seem to be associated with human tumors. The human Mps1 (hMps1) orthologue is required for normal mitotic progression due to its functions in centrosome duplication, the spindle checkpoint, and cytokinesis (15-18). It was shown that when hMps1 is removed from cells, it causes chromosomal irregularities during mitotic spindle formation and cytokinesis failure (15). Mps1 depletion also causes failures in centrosome duplication, although this function requires much lower levels of hMps1 (15). Mps1 is involved in many key cellular events, and its sequence and functions are conserved in many species from yeast to humans.

It was originally seen in mouse cells that Mps1 localizes to the centrosome (19). Mps1 specifically localizes to the centrioles, and its requirement for centrosome duplication suggests that targeting Mps1 to the centrosome is an important event. It has also been shown to localize to the kinetochores of chromosomes that are unattached to

the mitotic spindle (17, 19-21) and the anaphase promoting complex (16) in humans. It is important to identify exactly how Mps1 is targeted to these locations because it is most likely involved with its function at these locations. The domain responsible for localization to centrioles and kinetochores has been mapped to the N-terminus of Mps1 (16), but the precise factors of localization are not known. It is important that we determine specifically what region within the N-terminus is specific for centrosome localization because the levels of Mps1 at the centrosome are important; Mps1 is regulated by degradation at the centrosome (Kasbek et al., unpublished data).

Mps1 regulates the cell cycle through its interactions with kinetochores and centrioles, and its functions in the spindle checkpoint and centrosome duplication. As previously mentioned, centrosome defects have been seen in human tumors. Centrosome over-duplication, when more than one round of duplication is initiated per cell cycle, could very likely be causing the loss of genomic integrity seen in prostate (22) and breast (23) cancers. Since hMps1 is involved in regulating centrosome duplication, it is possible that defects in hMps1 could cause the centrosomal defects that might lead to these cancers. Therefore, it is important to know how hMps1 is regulated and how it is localized to the centrosome, because presumably if Mps1 cannot localize to the centrosome, then it cannot regulate centrosome duplication.

Chapter 2

Identification of the Centrosome Localization Domain

The first focus of the project was to identify the domain of hMps1 that is responsible for the physical localization to the centrosomes. We began looking in the N-terminus because it had already been shown to be involved in centrosome and kinetochore localization. Since Mps1 localization to the centrosome would likely have an important role as to how the protein functions, the centrosome localization domain (CLD) is likely to be a conserved region among species with known orthologues. The best candidate for investigation was a region of hMps1 near the N-terminus, from amino acids 58-172, that was almost completely conserved among mammals (Figure 2.1) and highly conserved among other vertebrate species (Figure 2.2). If a sub-clone made from that region of hMps1 was able to localize to the centrosome, then it would suggest that the conserved domain contained information that was sufficient for centrosomal localization. To test this, a sub-clone of hMps1 was made where green fluorescent protein (GFP) was fused to amino acids 58-172 of hMps1, transfected into mouse fibroblast cells, and localization of GFP was determined by fluorescence microscopy.

First, the conserved region on hMps1 was amplified using PCR primers from the pECE-GFP hMps1 template. The hMps1 148+(Kpn1) forward primer inserted a Kpn1 restriction site and the hMps1 536(-)(Xba1) reverse primer inserted an Xba1 restriction site, both restriction sites were positioned to insert the hMps1 sub-clone into the same reading frame as GFP for expression. Both the PCR product and the expression vector (pECE-GFP), which is a mammalian expression construct capable of expressing GFP fusion proteins from the Sv40 promoter, were digested with Kpn1 and Xba1. The

products were then ligated with T4 DNA ligase. Next, the plasmid was sequenced and verified to be correct. Afterwards, the pECE-GFP hMps1$_{58-172}$ plasmid was transfected into NIH 3T3 mouse fibroblast cells along with two controls, pECE-GFP and pECE-GFP hMps1. Indirect immunofluorescence (IIF) was performed using G-TU-88 (mouse anti-γ-tubulin) linked to a secondary antibody conjugated to AlexaFluor 594 to visualize centrosomes.

When analyzed by fluorescence microscopy it was found that pECE-GFP hMps1$_{58-172}$ was sufficient to promote the localization of GFP to centrosomes. This is not simply an artifact of overexpression, as GFP alone does not localize to centrosomes. About 90% of cells transfected with full length GFP-hMps1 showed the protein localized to centrosomes versus about 70% of cells transfected with GFP-hMps1$_{58-172}$ (Figure 2.3). This decrease in localization could be attributed to the fact that there were protein folding errors because we were using such a short region of hMps1. Also, it could be that the truncated version of hMps1 is less stable than full length. Nonetheless, since GFP alone does not localize to the centrosomes, we can conclude that the only way that GFP-hMps1$_{58-172}$ could have localized to the centrosomes was through an interaction between the conserved domain of hMps1 and the centrosome. Thus, the region of amino acids from 58-172 contains a signal that is sufficient for centrosome localization and is a true CLD, although it does not address whether that region is necessary for the ability of hMps1 to localize to the centrosome.

To investigate the necessity of the CLD for hMps1 localization to centrosomes, it is necessary to delete that region and test whether localization of GFP-tagged hMps1 still occurs. There are regions of conservation scattered throughout the CLD, so to begin

narrowing down the minimum localization domain we decided to delete the first half and the second half of the CLD separately. We designed primers to delete the whole region, from amino acids 57-112, and from amino acids 112-170. To do this, we designed primers that amplified the pECE-GFP hMps1 plasmid while excluding portions of the CLD. Primers were designed to have overlapping segments so that the PCR product would be re-circularized within bacteria to delete portions of the CLD. For the ease of primer design we defined the CLD as amino acids 57-170. The entirety of the CLD was deleted using the primers hMps1 Δ57-170 and hMps1 Δ57 Overlap. To delete the region from residues 57-112 the primers hMps1 Δ57-112 and hMps1 Δ57 Overlap were used. Finally, to delete the region from residues 112-170 the primers hMps1 Δ112-170 and hMps1 Δ112-170 Overlap were designed, but we have not successfully generated the construct. The plasmids obtained so far were sequenced and verified to be correct.

HeLa cells were then transfected with pECE-GFP $hMps1_{\Delta 57\text{-}170}$ and pECE-GFP $hMps1_{\Delta 57\text{-}112}$, using pECE-GFP and pECE-GFP hMps1 as controls. When analyzed by fluorescence microscopy both GFP $hMps1_{\Delta 57\text{-}170}$ and GFP $hMps1_{\Delta 57\text{-}112}$ proteins localize to the centrosomes (Figure 2.4). The GFP signals for the deletions were weaker compared to full-length hMps1, so it does not seem to localize as efficiently. A few things could potentially explain this. First, it is possible that there is another CLD, or at least some other domain that allows for a protein interaction that enhances centrosome localization. Another possibility involves a predicted dimerization domain in the C-terminus of hMps1. This domain could cause dimerization of endogenous hMps1 and the GFP-CLD deletions, leading to GFP signal at the centrosome regardless of the presence of a functional CLD on the deletions. This interaction could be abolished by designing an

siRNA experiment that selectively depleted endogenous hMps1 by targeting the CLD; therefore it could no longer dimerize with the GFP-CLD deletions. We would then be able to determine if the CLD was necessary for centrosome localization.

These experiments demonstrate that amino acids 58-172 contain a CLD. However, we have not yet been able to refine the CLD to its minimum domain. This region is sufficient for centrosome localization, but attempts to demonstrate necessity have not yet been completed. We will work on designing the siRNA experiment to determine the CLD's necessity as well as working to define the minimal CLD.

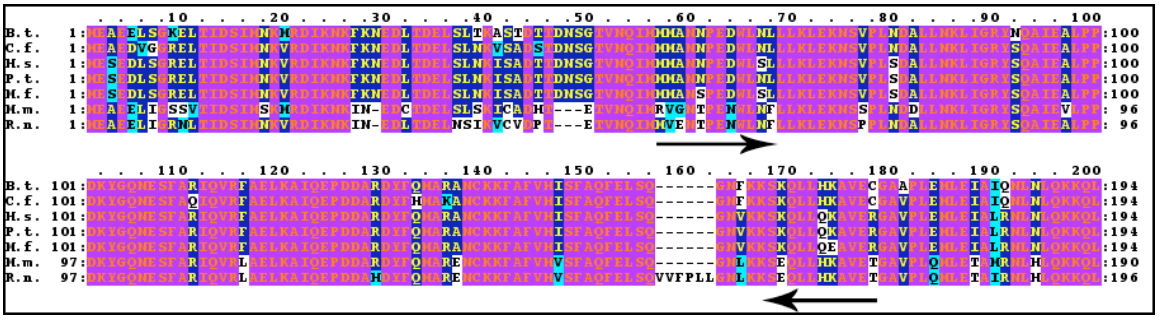

Figure 2.1 – In this list of 7 mammalian species there is a region that is almost completely conserved (pink) between amino acids 58-172 in humans.

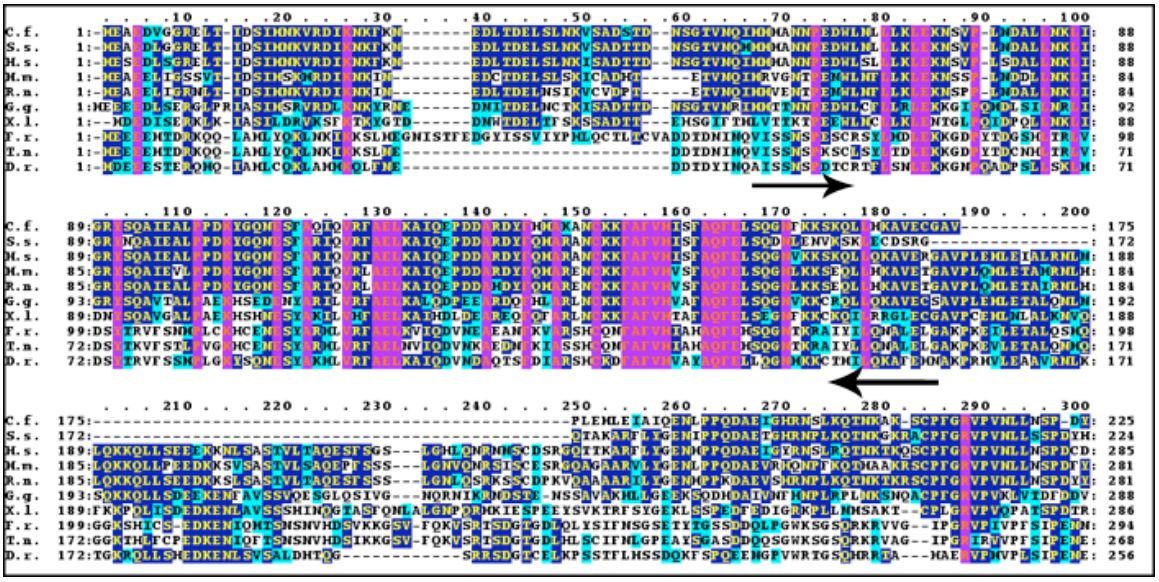

Figure 2.2 – This list of 10 vertebrate species contains an area of conserved residues (pink) in the region from amino acids 58-172 in humans.

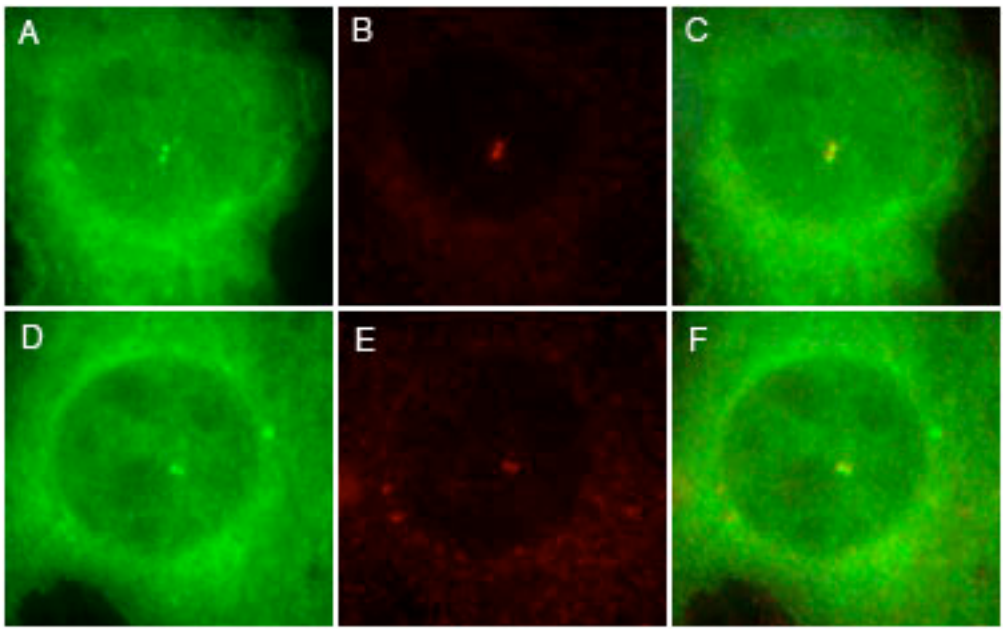

Figure 2.3 – Full length GFP-hMps1 (A) localizes to centrosomes (B) in 90% of transfected cells, merged image (C). GFP-hMps1$_{58\text{-}172}$ (D) localizes to the centrosomes (E) in 70% of transfected cells, merged image (F).

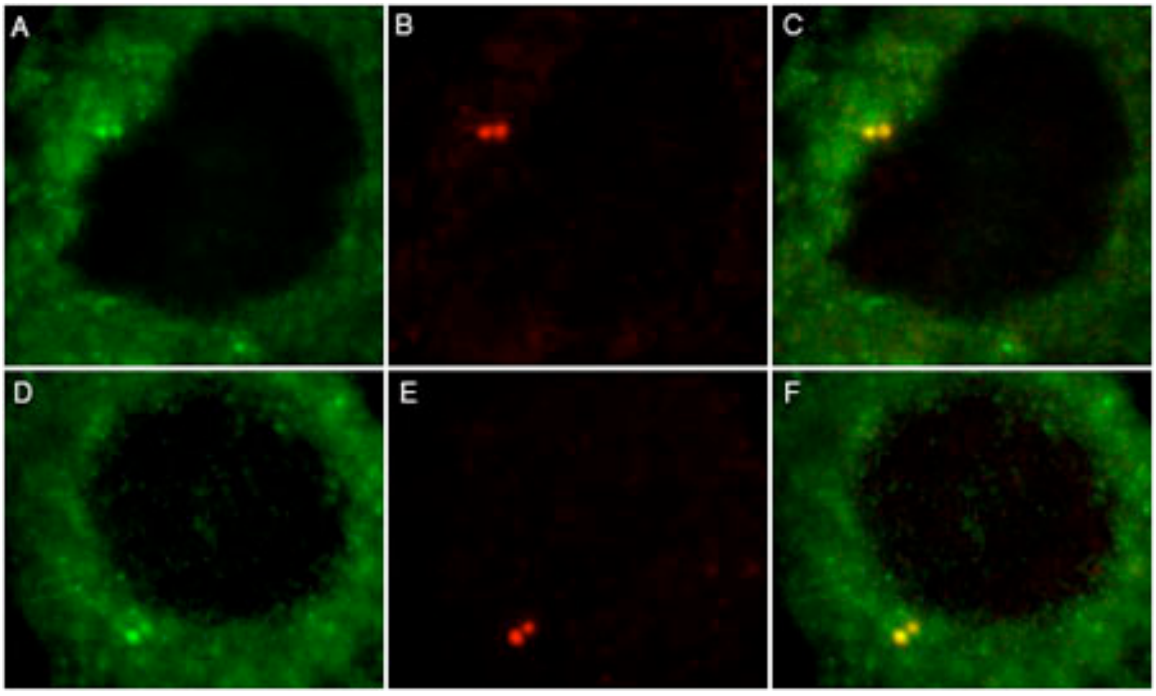

Figure 2.4 – GFP-hMps1$_{\Delta 57\text{-}170}$ (A) localizes to the centrosomes (B). Merged image (C). GFP-hMps1$_{\Delta 57\text{-}112}$ (D) localizes to the centrosomes (E). Merged image (F). Localization appears less bright than compared to wild type hMps1.

hMps1 148+ (Kpn1)	GGAACTGGTACCCAAATTATGATGATGG
hMps1 536(-) (Xba1)	ATTTCTAGAGGTACTGCTCCACG
hMps1 Δ57-170	CTGTTAACCAAATTATGGAACGTGGAGCAGTACC
hMps1 Δ57-112	CTGTTAACCAAATTATGCAAGTGAGATTTGCTG
hMps1 Δ57 Overlap	CATAATTTGGTTAACAGTTCCCGAGTTATCTGTAG
hMps1 Δ112-170	GTTTTGCTAGAATTCAAGAACGTGGAGCAGTACC
hMps1 Δ112-170 Overlap	TTGAATTCTAGCAAAACTCTCATTTTGGCC

Table 2.1 – Sequences of primers used.

Chapter 3
Effects of Tyrosine Phosphorylation on hMps1 Localization

The next focus for this project was to investigate the effects of tyrosine phosphorylation on the localization of hMps1. Within the conserved region there is a tyrosine residue at position 91 (Y91) that is completely conserved in vertebrate species. This is important for two reasons. First, the conservation of tyrosine suggests that it has a functional importance within Mps1. Second, cells often use phosphorylation events on tyrosine residues to regulate aspects of a protein's function and a tyrosine in the CLD might be involved in regulating hMps1 localization; so it is important to see whether phosphorylation of Y91 has some effect on the localization of hMps1 to the centrosome.

Mutagenic primers were designed that altered the sequence at position 91 of hMps1 to code for phenylalanine (Y91F) instead of tyrosine. Phenylalanine was chosen because it is structurally identical to tyrosine except that it has a hydrogen in place of the hydroxyl group and therefore cannot be phosphorylated (Figure 3.1). To aid in differentiating the wild type plasmid from the mutant plasmid the primers were also designed to create an Sml1 restriction enzyme site. Unfortunately, this site change altered the amino acid at position 92, replacing a serine with threonine, which is structurally and functionally similar. Threonine is present at this position in a few vertebrate species that were identified to have the conserved Y91, so this change will most likely conserve any structural or functional importance at that position.

Once the Y91F mutation was made via PCR on the pECE-GFP hMps1 plasmid, the CLD mutation was then sub-cloned into the pECE-GFP vector by the same method used to create the pECE-GFP $hMps1_{CLD}$ sub-clone from the previous experiment. The sequence was verified to be correct. The full-length hMps1 Y91F and $hMps1_{CLD}$ Y91F

mutants were then transfected into NIH 3T3 cells and the localization of GFP was analyzed by fluorescent microscopy. There was a significant decrease in the centrosome localization of these mutants compared to both the wild type full length and 58-170 sub-clone of hMps1. About 64% of the cells transfected with either pECE-GFP hMps1 Y91F or pECE-GFP $hMps1_{58\text{-}172}$ Y91F showed proteins localized to the centrosomes (Figure 3.2). This decrease in localization is significant between full-length hMps1 versus Y91F, but there is no significant change in the percentage of localization between the CLD sub-clones. However, there was a decrease in intensity in the Y91F mutants versus wild type, which suggests that tyrosine phosphorylation has some effect on the efficiency of centrosome localization, but does not abolish it completely.

We then decided to investigate further and see the consequences when there is constitutive phosphorylation of this residue. To do this we obtained three different tyrosine phosphatase inhibitors. We transfected HeLa cells with pECE-GFP $hMps1_{CLD}$ and used pECE-GFP $hMps1_{CLD}$ Y91F for comparison. Both cells were treated with the tyrosine phosphatase inhibitors for 1 or 2 hours, untreated cells were used as a control. After treatment, cells were collected and the hMps1 protein was purified by immunoprecipitation on magnetic beads using anti-GFP antibodies. Proteins were analyzed by SDS-PAGE and Western Blotting. Anti-GFP antibodies were used to visualize GFP-tagged hMps1 and an anti-phosphotyrosine antibody was used in an attempt to visualize tyrosine phosphorylation. GFP-hMps1 signal was detectable in untreated cells and after 1 hour of tyrosine phosphatase inhibitor treatment, but was absent after 2 hours of treatment. GFP-hMps1 Y91F, however, was still detectable after 2 hours of treatment. So it is interesting to note that wild type hMps1, which should exhibit

constitutive phosphorylation on Y91 due to the tyrosine phosphatase inhibitors, is absent from the 2-hour treatment while the Y91F mutant, which is unable to be phosphorylated, is present at the 2-hour time-point (Figure 3.3). This data suggests that constitutive phosphorylation of Y91 causes the protein to be degraded. This is consistent with the data that Mps1 is degraded at the centrosome; since we hypothesize that constitutive Y91 phosphorylation would cause an increase in centrosome localization of hMps1. Unfortunately, the anti-phosphotyrosine antibody gave a very non-specific signal, so we could not compare phosphorylation levels.

We again transfected HeLa cells with full-length wild type and Y91F hMps1 plasmids and treated with the tyrosine phosphatase inhibitors to see what happens to hMps1 localization at the cellular level. We performed IIF as described above on untreated controls and after 1 and 2 hours of treatment. Wild type hMps1 appeared to be highly localized to the nucleus when treated with the tyrosine phosphatase inhibitors while the Y91F mutant was absent from the nucleus (Figure 3.4). It appears that constitutive Y91 phosphorylation causes nuclear accumulation of hMps1, which is difficult to correlate with the degradation of the hMps1 protein as seen by Western blot. Since these two experiments were done separately, we plan to repeat them side-by-side and try to draw conclusions from the results. However, the results could potentially be explained by the fact that the addition of tyrosine phosphatase inhibitors might have a larger effect on cells, since some very important cellular events are regulated by tyrosine phosphorylation.

In these experiments we investigated the effects of Y91 phosphorylation. We have demonstrated that a lack of Y91 phosphorylation causes a decrease in the levels of hMps1

present at the centrosome. Also, constitutive phosphorylation on Y91 causes the wild type protein to be degraded after two hours, while $hMps1_{CLD}$ Y91F is still present after two hours when viewed on a Western blot. At the cellular level, constitutive phosphorylation on Y91 causes $hMps1_{CLD}$ to be concentrated in the nucleus while $hMps1_{CLD}$ Y91F is concentrated in the cytosol. From this we can conclude that hMps1 localization is regulated by Y91 phosphorylation, but we cannot conclude the exact effects it has on localization.

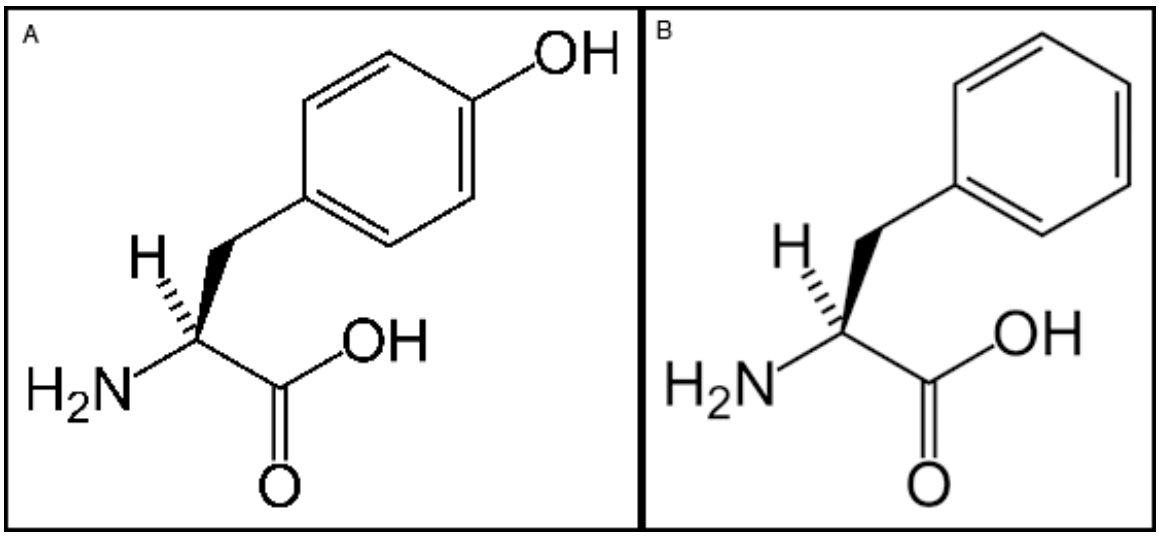

Figure 3.1 – Tyrosine (A) is structurally identical to phenylalanine (B) except that phenylalanine lacks the 4' hydroxyl group that allows for tyrosine to be phosphorylated.

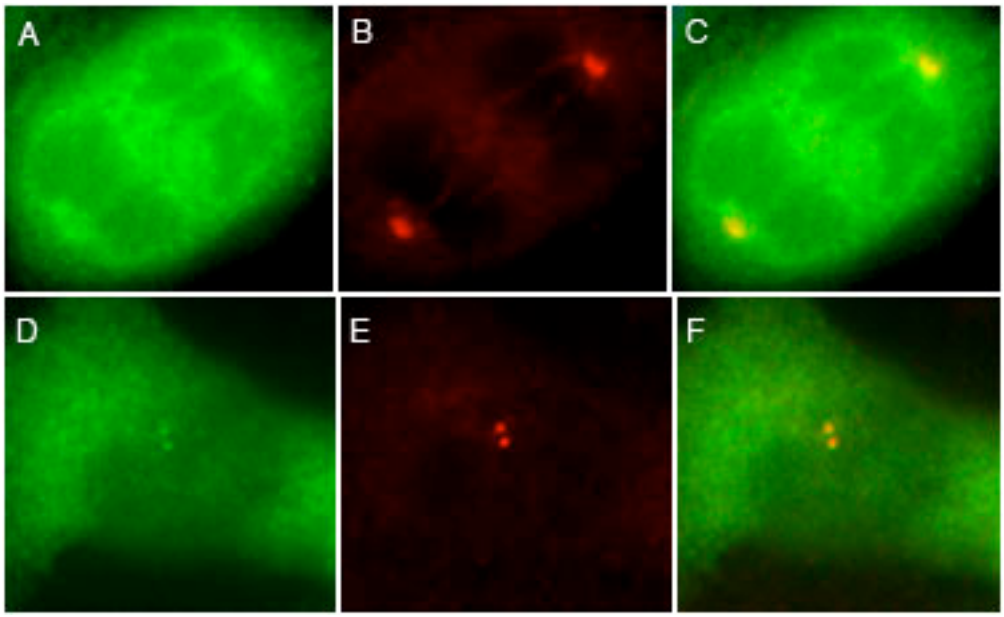

Figure 3.2 – Full length GFP-hMps1 Y91F (A) appears to localize faintly to centrosomes (B), merged image (C). GFP-hMps1$_{58\text{-}172}$ Y91F (D) faintly localizes to the centrosomes (E), merged image (F). Both proteins localized to the centrosome in about 64% of transfected cells, but GFP-signals were weaker than wild type.

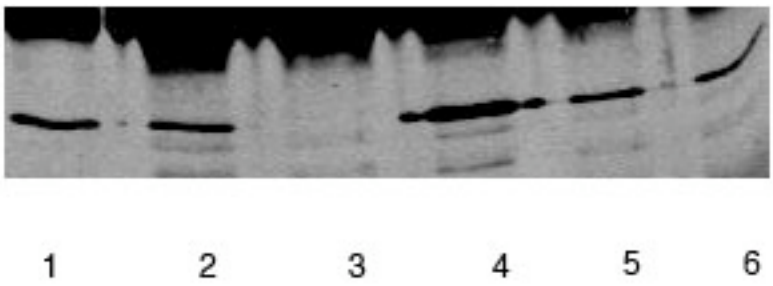

Figure 3.3 – Lanes 1 and 4 are untreated $hMps1_{CLD}$ Y91 and $hMps1_{CLD}$ Y91F respectively. Lanes 2 and 5 are $hMps1_{CLD}$ Y91 and $hMps1_{CLD}$ Y91F after 1 hour of tyrosine phosphatase inhibitor treatment. Lanes 3 and 6 are $hMps1_{CLD}$ Y91 and $hMps1_{CLD}$ Y91F after 2 hours of treatment. $hMps1_{CLD}$ Y91 is absent after 2 hours of treatment.

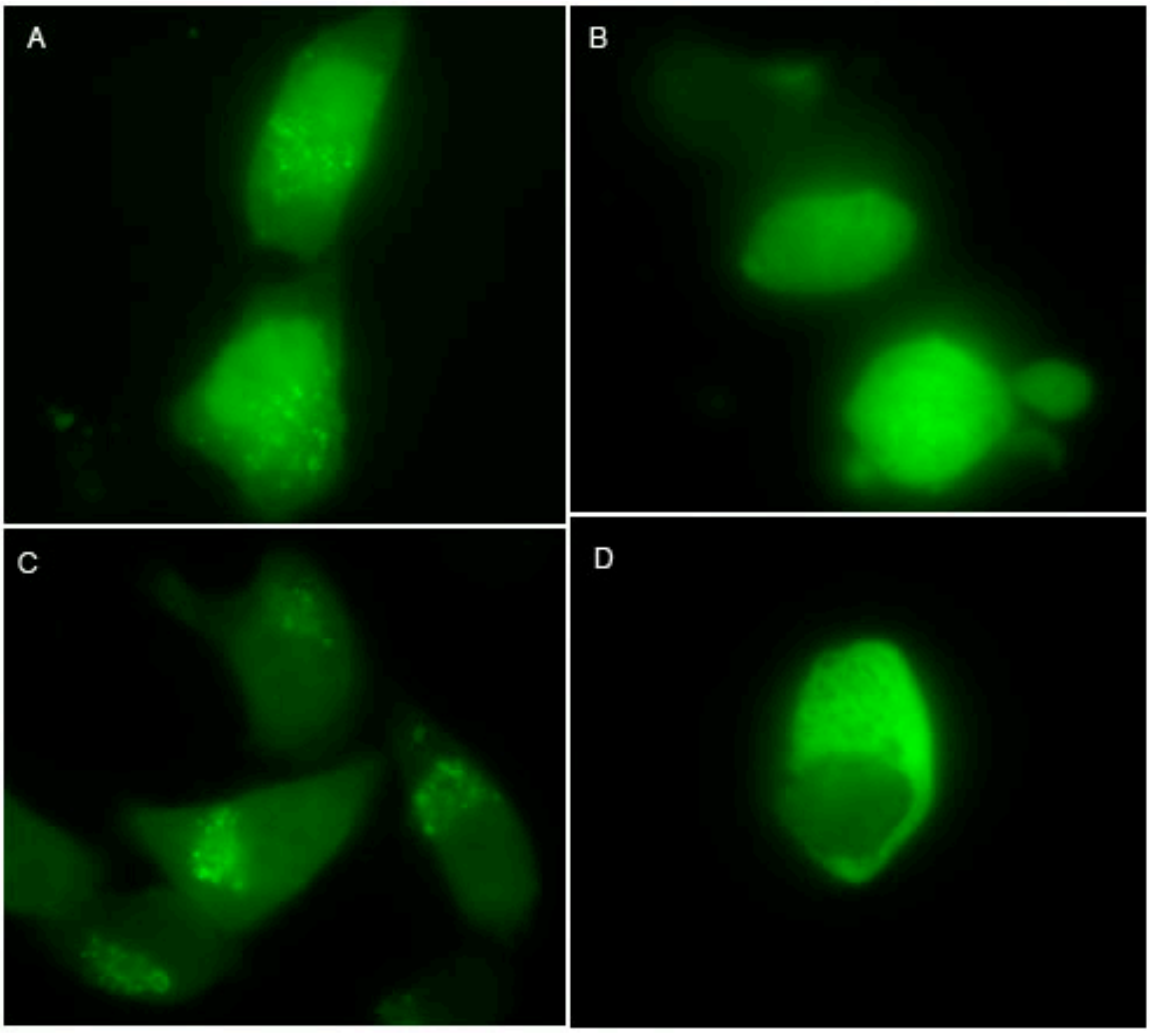

Figure 3.4 – GFP-tagged wild type $hMps1_{CLD}$ is visible in the nucleus in untreated cells (A) and is concentrated in the nucleus after 2 hours of treatment (B). GFP-$hMps1_{CLD}$ Y91F is absent from the nucleus in untreated cells (C), and is concentrated in the cytosol after 2 hours of treatment (D).

Chapter 4

Identification of CLD Binding Partners

The final goal of this study was to identify proteins that interact with the CLD and allow for the centrosome localization of hMps1. It is important that we identify what protein or proteins assist in targeting hMps1 to the centrosome. Also, the presence of the conserved Y91 residue suggests that the CLD might be regulated by phosphorylation, which would mean that the CLD would interact with a protein kinase. Because the CLD directs GFP to the centrosome, we hypothesize that the CLD binds to some centrosomal protein or proteins. To identify these proteins we decided to use the two-hybrid system, which utilizes fusion proteins, reporter genes, and auxotrophic yeast strains to detect protein interactions.

The two-hybrid system works by expressing the transcription factor for the GAL4 promoter as two separate proteins. First, the DNA-binding domain (DB) of the transcription factor, which is responsible for interacting with the correct DNA promoter, is expressed as a fusion protein with a gene of interest (DB-X). The activation domain (AD) of the transcription factor, which normally recruits the polymerase for transcription, is fused to a cDNA library of different proteins (AD-Y). Normally these two domains are part of the same protein, so that GAL4 recruits the polymerase directly to the DNA it is supposed to transcribe (Figure 4.1). When these two domains are expressed separately, the polymerase can no longer be recruited directly to the right DNA for proper transcription. In the two-hybrid system there is a reporter gene under the control of the GAL4 promoter. For example, with the URA3 reporter gene, cells cannot grow when the gene is not transcribed. Transcription of the reporter gene only occurs when there is an

interaction between the CLD and a protein from the cDNA library, which allows cells to grow and be selected. This interaction restores the interaction of the two domains of GAL4, and thus restores the recruitment of the polymerase to the reporter gene (Figure 4.2). Transformants can be screened for cells that grow in the absence of histidine (HIS3+), uracil (URA3+), and also exhibit β-galactosidase activity (lacZ+). This is a useful technique because it can quickly screen a very large pool of proteins for interactions with a gene of interest.

To begin, we cloned the $hMps1_{58-172}$ construct into the pDEST 32 destination vector, a plasmid that has been designed to express the GAL4 DNA binding domain as a fusion with any gene of interest (e.g. DB-CLD) and contains the LEU2 selectable marker. Next, the MaV203 yeast strain designed for the system was transformed with the DB-CLD plasmid. We then screened for DB-CLD transformants, which could grow in the absence of leucine, and transformed them with the AD-Y plasmid library, which contained the TRP1 selectable marker. Yeast cells that contained both plasmids were able to grow in the absence of leucine and tryptophan. Positive transformants for both plasmids were then transferred to a set of master plates. Over 1,000,000 transformants were obtained initially. MaV203 yeast strains have the ability to produce low basal levels of histidine, but this biosynthesis can be inhibited with the addition of 3-amino-1,2,4-triazole (3AT) in order to increase the sensitivity of the screen. We determined that a 25mM concentration of 3AT was needed to effectively inhibit the basal levels of histidine biosynthesis. From the transformants, 1,800 were isolated with the ability to grow in the absence of histidine. Replicas were made from the master plate onto YC media lacking leucine, tryptophan, and histidine with the addition of 3AT. 120 screened positive for the

HIS3 reporter while 62 out of the 120 screened positive for the URA3 reporter. Finally, five transformants screened positive for the lacZ reporter by testing for the presence of β-galactosidase, which reacts with X-Gal and turns the yeast colonies blue after incubation. Four colonies exhibited a strong blue color after only 20 minutes, while one colony exhibited a weak blue color after 2 hours; the remaining 57 colonies were colorless after 2 hours. These five plasmids were isolated and the inserts were sequenced and compared to known genes with a BLAST search. The identities of the four strongly interacting proteins were VDAC3 (voltage-dependant anion-selective channel 3), α-6-tubulin, translation elongation factor 1-gamma, and a previously uncharacterized clone from chromosome 8. The weakly interacting protein was identified to be ribosomal protein L13a. The insert identified as the chromosome 8 clone also matched a large number of other clones, therefore it is probably not a true protein interaction. In addition, ribosomal protein L13a was identified in another two-hybrid screen from a different domain of hMps1, which suggests that it is also not a true protein interaction.

From the remaining proteins identified, VDAC3 and α-6-tubulin are potentially the most interesting. α-tubulin is a main component of microtubules and microtubules are organized by the centrosome. Also, centrioles are composed of microtubules and Mps1 localizes to centrioles. It would not be difficult to imagine that hMps1 might bind directly to the centrioles via an interaction with α-tubulin at the centriole. However, we have not further investigated this potential interaction since we have been investigating VDAC3. VDACs' are a family of voltage-dependant anion-selective channels. Normally channel proteins are embedded in membranes; however, VDAC3 has been identified in the outer dense fiber of bovine sperm flagella (24). In addition, the outer dense fiber protein ODF2,

originally identified in sperm flagella, has been identified at the centrosome and appears to associate with the mother centriole (25). The outer dense fibers are located on the outside of the axoneme (26), which is somewhat analagous to the distal appendage of the mother centriole. Also, the doublet microtubule structure of a flagellum is very similar to a centriole. These findings support the possibility that VDAC3 might be incorporated into the structure of the centrosome and could potentially be a docking point for hMps1. It is possible that VDAC3, like ODF2, is present at the centrosome and potentially associates specifically to the mother centriole. All these facts suggest that VDAC3 might actually be at centrosomes where it could potentially interact with Mps1.

To examine whether VDAC3 is a true centrosome protein, we examined VDAC3 localization. We obtained a cDNA clone of VDAC3 and cloned it into the pECE-GFP vector as described previously for the CLD. We then transfected the pECE-GFP VDAC3 plasmid into HeLa cells and performed IIF as described previously to see where VDAC3 might be localized. We have found that GFP-VDAC3 does indeed localize to the centrosome (Figure 4.3). Also, in cells that have already duplicated their centrosome, the GFP-signal only associates with one of the centrosomes, which is consistent with our hypothesis that VDAC3 might be associated with the mother centriole (Figure 4.4). Although these results show that over-expressed GFP-tagged VDAC3 can localize to the centrosome, it does not address whether the endogenous protein also associates with the centrosome. We have obtained an antibody against VDAC3 and are currently testing the best conditions to stain VDAC3 in IIF experiments.

In this experiment we utilized the two-hybrid system and identified five potential binding partners for the CLD. We have further investigated one of the identified proteins,

VDAC3, and have shown that GFP-tagged VDAC3 localizes to the centrosome. Further investigation is necessary to determine whether the endogenous protein is indeed a novel centrosomal protein. We will also have to test whether VDAC3 and Mps1 interact *in vitro*. The remaining proteins also need to be investigated further to validate their involvement with the CLD.

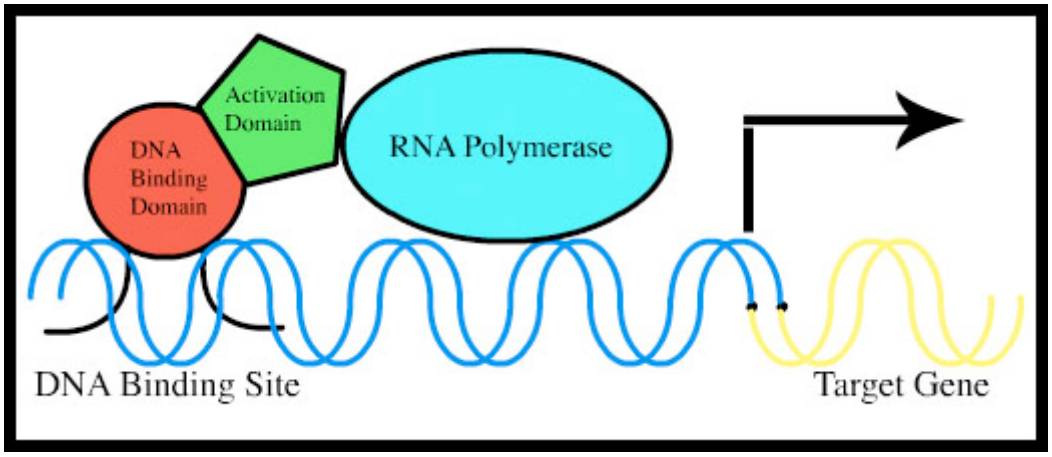

Figure 4.1 – The DNA binding domain and the activation domain are normally expressed together in order to recruit the RNA polymerase and promote transcription of the target gene.

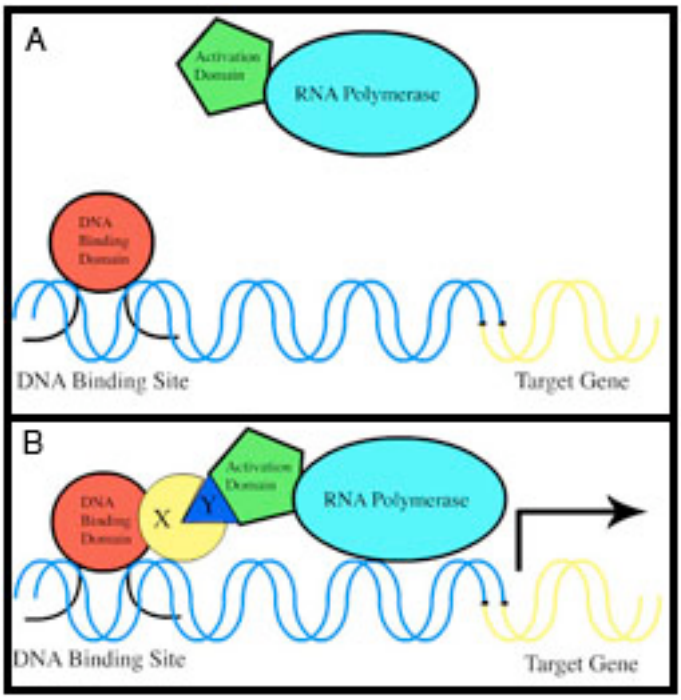

Figure 4.2 – When the DNA binding domain and the activation domain are expressed separately their interaction is abolished and the target gene is not transcribed (A). When the DNA binding domain is expressed as a fusion with the CLD and the activation domain is expressed as a fusion with a library protein that interacts with the CLD, function is restored and the target gene is transcribed (B).

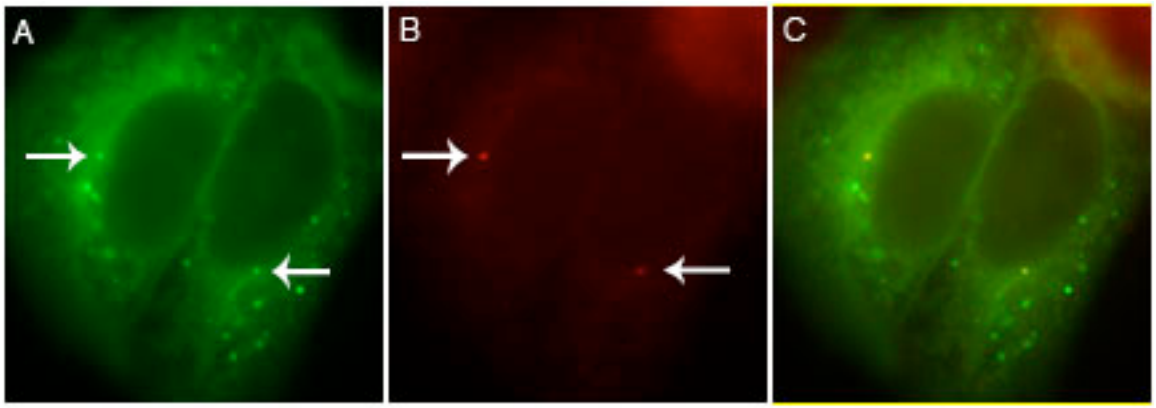

Figure 4.3 – GFP-VDAC3 (A) colocalizes with the centrosomes (B). Merged image (C).

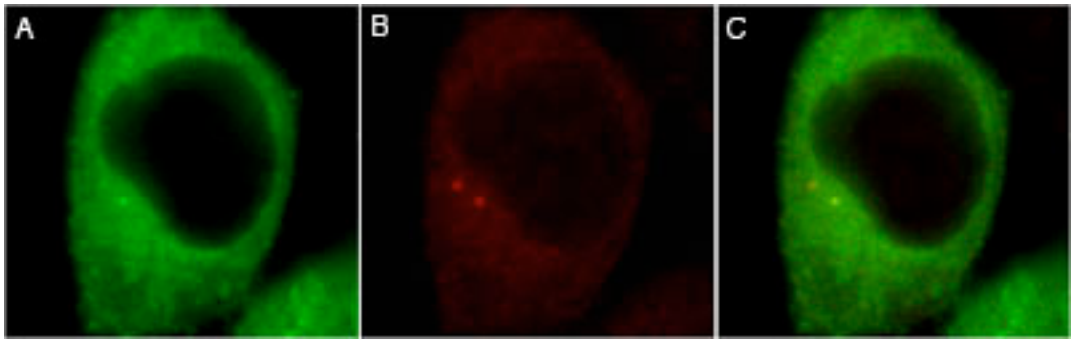

Figure 4.4 – GFP-VDAC3 (A) appears to localize specifically to one centrosome (B). Merged image (C).

pDEST 32	Contains LEU2 selectable marker
pPC86	Contains TRP1 selectable marker
HIS3 reporter gene	Allows cells with DB-X:AD-Y interaction to grow in the absence of histidine
URA3 reporter gene	Allows cells with DB-X:AD-Y interaction to grow in the absence of uracil
lacZ	Encodes β-galactosidase which is an enzyme that cleaves X-gal and causes cells containing β-galactosidase to be blue
YPAD	Rich medium that allows for growth of untransformed cells
YC –leu	Selectable medium that only allows for the growth of cells containing the LEU2 marker
YC –leu -trp	Selectable medium that only allows for the growth of cells containing both the LEU2 and TRP1 markers
YC –leu –trp –his +25mM 3AT	Selectable medium that only allows for the growth of cells containing both the LEU2 and TRP1 markers that also exhibit an interaction between DB-X:AD-Y
YC –leu –trp -ura	Selectable medium that only allows for the growth of cells containing both the LEU2 and TRP1 markers that also exhibit an interaction between DB-X:AD-Y

Table 4.1 – Information on selectable markers, reporter genes, and media used in the two-hybrid system.

Chapter 5

Discussion

One important aspect of hMps1 is that it is localized to the centrosomes during the entire cell cycle. While it is believed that its localization to the centrosome is a required event for duplication, we don't actually know for sure. This study has potentially uncovered a tool that could help answer that question. One of the biggest successes of this project is that we now know the identity of at least one domain responsible for the centrosome localization of hMps1. While it has not yet been refined, we have shown that this domain is sufficient for the centrosome localization of hMps1. We will have to determine whether or not it is necessary for localization in order for it to truly be considered the only CLD in hMps1. If it also turns out to be necessary it will mean that there is no other method for hMps1 to localize to the centrosome. If that is the case then we will have created a tool to investigate whether or not centrosome localization is necessary for its function in regulating duplication.

We are currently designing an siRNA experiment that would specifically deplete endogenous hMps1 while exogenous GFP-tagged hMps1$_{\Delta 57\text{-}170}$ would remain in the system. siRNA experiments utilize short RNA strands that are specific to an mRNA sequence, and interfere with the translation of the mRNA and stop protein production. We could target the siRNA to the sequence of the CLD, so endogenous protein, which contains the CLD, would not be translated while the exogenous protein, which lacks the CLD, would be translated. This experiment would allow us to potentially answer two questions. If hMps1 is able to dimerize with GFP-hMps1$_{\Delta 57\text{-}170}$ artificially, then we should see a decrease in localization of the GFP to centrosomes in siRNA transfected

cells. However, if GFP can still co-localize with the centrosome at similar levels, we have to look into the presence of alternate CLDs; something functionally important could very well have a redundant mechanism. If $hMps1_{\Delta 57\text{-}170}$ is unable to localize to the centrosome, we can see if the centrosome has been duplicated. If the centrosome is not being duplicated, it would strongly suggest that hMps1 centrosome localization is required for its function in centrosome duplication. We will also need to continue to work on identifying the minimum localization domain.

Another feature of the CLD identified by this study is that tyrosine phosphorylation is involved in its regulation. While we have not been able to clearly define the effects, the inability of the Y91 residue to be phosphorylated definitely decreases the efficiency of hMps1 localization based on our results. We are currently working on creating mutations that mimic phosphorylation by replacing Y91 with aspartic or glutamic acid. One potential problem is that those mutations are generally used to mimic phosphorylation of serine and threonine; the structure of tyrosine is drastically different and these mutations might not properly mimic its structure. However, these mutations are one of the best methods to address the question without using tyrosine phosphatase inhibitors. It will also be interesting to further investigate the experimental results suggesting the nuclear localization of constitutively phosphorylated Y91.

Finally, in this study we have potentially identified a novel centrosomal protein. VDAC3 has never been shown to localize to the centrosomes. We must show that endogenous VDAC3 actually localizes to the centrosome and it is not a neomorphic function of GFP-tagged VDAC3 that causes centrosome localization. In addition, we

must also determine if VDAC3 actually interacts with hMps1 by co-immunoprecipitation or an *in vitro* binding assay. We also must investigate the other four proteins that were identified as potential hMps1 binding partners.

This study has found the answers to a handful of questions; but as with any experiment, it has also raised questions for future experiments. We have identified a new domain of hMps1, identified a potential mechanism for its regulation, and identified a handful of proteins that it might interact with. We still need to identify the minimal localization domain, investigate the full effects of Y91 phosphorylation, and we now have a list of proteins to investigate. The functions of hMps1 must be studied in depth in order to draw any conclusions about its functions involving the centrosome and any potential functions in human disease. With all that is known about hMps1, it is not unlikely that could be an important aspect in human disease. It will be exciting to see what new information is gathered in the future.

Chapter 6

Materials and Methods

Cell Culture and Transfections

All cells were incubated in DMEM (Gibco) and transfected 24 hours after a 1:10 passage. Transfections were performed using the Qiagen Effectine transfection reagent. 400 ng DNA was pre-incubated with buffer EC, then 3.2 μL of Enhancer was added for a final volume of 100 μL, mixed, and incubated for 5 minutes at room temperature. Either 10 μL (HeLa) or 2 μL (NIH 3T3) of Effectine reagent was added, mixed, and incubated for 10 minutes. After incubation, the mixture was added to 600 μL of media and added to cells in 1.2 mL of medium. After a 24 hour incubation at $37^{\circ C}$ with 5% CO_2, cells were either collected or coverslips were fixed. The tyrosine phosphatase inhibitor treatment was administered 24 hour post-transfection and cells were collected either 1 or 2 hours later. Phenylarsine oxide (Calbiochem), L-p-bromotetramisole oxalate (Biomol), and benzylphosphonic acid – $(AM)_2$ (Biomol) were at a final concentration of 1mM in these experiments, and were also added to the lysis buffer at 1mM.

Antibodies

The G-TU-88 (mouse anti-γ-tubulin) antibody (Sigma) was used at a 1:1000 dilution, Hoechst-33342 (Sigma) was used at a 1:1000 dilution. The VDAC3 (polyclonal chicken anti-VDAC3) antibody (Abcam Inc.) was tested using a 1:500 dilution. AlexaFluor 594 conjugated anti-mouse antibody (Molecular Probes) and AlexaFluor 488 conjugated anti-chicken antibody (Molecular Probes) were used at a 1:1000 dilution.

Immunofluorescence

All immunofluorescence experiments were performed using an Olympus IX81 microscope. Coverslips were fixed in 0.2mL of a 4% paraformaldehyde / 0.1% Triton X-100 solution for 10 minutes. Cells were blocked in IIF Blocking Buffer (5.0% FBS, 0.1% Triton X-100, 0.2M Glycine, 1xPBS) for 1 hour, washed with 1xPBS/0.5mM $MgCl_2$, primary antibodies were added and incubated for 1 hour at room temperature, coverslips were washed again, secondary antibodies were added and incubated for 1 hour at room temperature, then coverslips were mounted onto slides with Citifluor Mountant Medium (Ted Pella Inc.).

Immunoprecipitation

Proteins were purified using Protein G magnetic beads (Invitrogen). 200μL of crude cell extract was pre-incubated with 20μL of beads and mixed at 4°C for 1 hour. 2μg of mouse anti-GFP antibody (Molecular Probes) was pre-incubated with beads and mixed at 4°C for 1 hour, and then the cell extract was added and incubated at 4°C for 1 hour. The beads were washed with lysis buffer, 4xSDS sample-loading buffer was added, the mixture was boiled for 5 minutes, and then loaded on an SDS-PAGE gel.

Two-Hybrid Analysis

A fragment containing amino acids 58-172 of hMps1 was cloned into Kpn1 and Xba1 sites of pDEST 32 (Invitrogen) to produce a fusion with the GAL4 DNA binding domain. The pPC86 pre-made cDNA library (Invitrogen) was used to produce a fusion with the GAL4 activation domain. The yeast strain MaV203 was used. Plasmid DNA was transformed using 700μL 40% PEG-3350/1xLiAC/1xTE solution and 1μg of plasmid mixed with 100μg denatured sheared salmon sperm and 100μL of yeast suspension, then

incubating at 30°C for 30 min followed by addition of 88μL DMSO and heat shocking for 7 min at 42°C. Cells were then washed with 1xTE and plated. Yeast transformations followed the Hybrid Hunter (Invitrogen) protocol. Tranformants were selected using dropout media. All procedures were done according to the ProQuest™ Two-Hybrid System with Gateway™ Technology manual.

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